TRADE SECRET

FINAL REPORT

Study Title

H-28072: In Vivo Micronucleus and Chromosome Aberration Assay in Mouse Bone Marrow Cells

Testing Guideline

US EPA Health Effects Test Guidelines, OPPTS Guideline 870.5395 and 870.5385 (1998)
EC Commission Directive 2000/32/EC Annex 4A-B10 No. L 136 (2000)
OECD Guidelines for Testing of Chemicals Section 4: Health Effects, No. 474 and 475 (1998)

Authors

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Study Completion Date

10 October 2007

Testing Facility

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for

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BioReliance Study Number

AC03GE.123108.BTL

Work Request Number

17319

Service Code

553 and 572

STATEMENT OF COMPLIANCE

Study No. AC03GE.123108.BTL was conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 160 and 40 CFR 792, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

> The test substance dose formulations were not analyzed for concentration/homogeneity or stability by the testing facility or the Sponsor. However, animal exposure to the appropriately prepared formulations is confirmed by clinical signs and mortality observed during the course of the definitive study.
>
> The positive control substance dose formulation (Cyclophosphamide monohydrate) was not analyzed by the testing facility or the Sponsor; however, the accuracy of preparation and stability of the formulation was demonstrated by acceptable results that met the criteria for valid test.

Applicant/Sponsor: E.I. du Pont de Nemours and Company
Newark, DE 19714-0050, USA

BioReliance Study Director: Ramadevi Gudi — 10 Oct 2007
Ramadevi Gudi, Ph.D. — Date

BioReliance Study Management: [signature] — 10 Oct 2007
Date

Applicant/Sponsor: _____ _____
DuPont Representative — Date

Quality Assurance Statement

Study Title: H-28072: IN VIVO MICRONUCLEUS AND CHROMOSOME ABERRATION ASSAY IN MOUSE BONE MARROW CELLS

Study Number: AC03GE.123108.BTL

Study Director: Ramadevi Gudi, Ph.D.

Quality Assurance performed the inspections listed below for this study. Verification of the study protocol was also performed and documented by QA. Procedures, documentation, equipment records, etc., were examined in order to assure that the study was performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Compliance Programme, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice, and to assure that the study was conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

Inspect On: 20-Jul-07 - 20-Jul-07 To Study Dir 20-Jul-07 To Mgmt 24-Jul-07
Phase: Staining of Slides

Inspect On: 08-Aug-07 - 10-Aug-07 To Study Dir 10-Aug-07 To Mgmt 15-Aug-07
Phase: Draft Report and Data Audit

Inspect On: 10-Oct-07 - 10-Oct-07 To Study Dir 10-Oct-07 To Mgmt 10-Oct-07
Phase: Draft to Final Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Allison Schaefer 10/OCT/07

Allison Schaefer, B.S. DATE
QUALITY ASSURANCE

CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Issued by Study Director: Ramadevi Gudi 10 Oct 2007

Ramadevi Gudi, Ph.D.
BioReliance

Date

Approved by
Study Sponsor:

E. Maria Donner, Ph.D.
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TABLE OF CONTENTS

Page

STUDY INFORMATION

Substance Tested:
- HFPO Dimer Acid Ammonium Salt
- 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propionic acid, ammonium salt
- 62037-80-3 (CAS Number)
- H-28072

Haskell Number: 28072

Composition:
82.6% Ammonium 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propionate*
13.9% Water
3.5% Ammonium
0.41% Organic Impurities

* Note: The Ammonium-2,3,3,3-tetrafuoro-2-(heptafluoropropoxy) propionate component (HFPO Dimer ammonium salt) contains 0.1 ppm HFPO trimer ammonium salt.

Purity: See composition, above

Physical Characteristics: Clear and colorless concentrated aqueous solution

Stability: The test substance appeared to be stable under the conditions of the study; no evidence of instability was observed.

Study Initiated/Completed: June 11, 2007/ (see report cover page)

Experimental Start/Termination: June 18, 2007/July 27, 2007

1.0 SUMMARY

The test substance, H-28072, was tested for genotoxic (clastogenic) potential by conducting the micronucleus and chromosome aberration assays. The assay was performed in two phases. The first phase, the dose range-finding phase, was designed to assess the toxicity of the test substance and to set dose levels for the definitive micronucleus study. The dose range-finding phase consisted of a pilot toxicity study followed by a toxicity study. The second phase, the definitive micronucleus study, was designed to evaluate the potential of the test substance to increase the incidence of chromosome aberrations in bone marrow cells (chromosome aberration assay) and the incidence of micronucleated polychromatic erythrocytes (micronucleus assay) in bone marrow of male and female ICR mice.

Based on a request by the Sponsor, sterile water was used as the test substance vehicle. All dose formulations were adjusted for purity of the test substance of 84.5% using a correction factor of 1.18. After completion of the experimental phase of the study, the Sponsor provided a Certificate of Analysis with the test substance purity of 82.6%. The differences between dose levels achieved using a correction factor of 1.18 and recalculated dose levels using a correction factor of 1.21 were in the range of 2.5%. Therefore the differences were considered negligible and the dose levels were not corrected for new provided purity. In the definitive phase of the study, sterile water was used as the vehicle (negative) control substance and cyclophosphamide monohydrate (CP), at a dose of 50 mg/kg, as the positive control substance. In both phases of the study, test or control substances were administered at a dose volume of 10 mL/kg body weight by a single oral gavage.

In the pilot toxicity study, two male mice each were exposed to H-28072 at 1, 10, 100 or 1000 mg/kg while five male and five female mice were exposed to H-28072 at 2000 mg/kg. Mortality was observed in 4/5 males and 4/5 females at 2000 mg/kg. Piloerection was seen in 1/2 males at 1 mg/kg, all males at doses ≥ 10 mg/kg and all females at 2000 mg/kg. Lethargy and cool to the touch were noted in all males at 1000 and 2000 mg/kg and all females at 2000 mg/kg. No appreciable changes occurred in the mean group animal body weights of males at doses ≤ 100 mg/kg. Appreciable reductions in the mean group animal body weights of up to 10.9% and 8.6% occurred in males at 1000 and 2000 mg/kg, respectively, and of up to 12.6% in females at 2000 mg/kg. In order to further assess toxicity of the test substance, a toxicity study was performed.

In the toxicity study, male and female mice (5/sex/group) were exposed toH-28072 at 1200, 1400, 1600 or 1800 mg/kg. Mortality was observed in 1/5 males at 1400 mg/kg, 2/5 males and 1/5 females at 1600 mg/kg and 3/5 males and 2/5 females at 1800 mg/kg. Lethargy and piloerection were seen in all males and all females at all doses. No appreciable changes in the mean group animal body weights of males or females occurred at any of the doses. Based upon these results, the high dose for the definitive micronucleus study was set at 1300 mg/kg, which was estimated to be the maximum tolerated dose.

The assay consisted of seven groups, each containing 5 male and 5 female mice. Mice in five of these groups were treated either with the controls (vehicle or positive) or with H-28072 at 325, 650 or 1300 mg/kg and were euthanized 24 hours after treatment. Mice in the other two groups were treated either with the vehicle control or H-28072 at 1300 mg/kg and were euthanized 48 hours after treatment. An additional group of 5 male and 5 female mice were treated with H-28072 at 1300

mg/kg to be used as replacement animals for the high dose in the event of mortality. Animals were observed for signs of toxicity during the course of the study. From each animal, at the time of euthanasia, bone marrow from one femur was collected and processed for micronucleus analysis and the bone marrow from the other femur was processed for analysis of chromosome aberrations.

Slides for the micronucleus analysis were stained with May-Gruenwald-Giemsa stain, and bone marrow cells [polychromatic erythrocytes (2000 PCEs/animal)] were examined microscopically for the presence of micronuclei (micronucleated PCEs; MPCEs). A statistically significant difference in the incidence of micronucleated PCEs in the test substance-treated groups relative to the concurrent vehicle control groups was determined based on the Kastenbaum-Bowman Tables (binomial distribution) for $p \leq 0.05$. The incidence of micronucleated PCEs and the ratio of PCEs to total erythrocytes (PCEs/ECs ratio) per each group served as indication of test substance clastogenicity and cytotoxicity, respectively.

Slides for chromosome aberration analysis were stained with Giemsa stain, and metaphase cells were examined and scored for chromatid-type and chromosome-type aberrations. In addition, the mitotic index was recorded as the percentage of cells in mitosis based upon 1000 cells counted per animal. A statistically significant difference in the incidence of structural and numerical chromosome aberrations in the test substance-treated groups relative to the concurrent vehicle control groups was determined based on the Fisher's exact test for $p \leq 0.05$.

Mortality was observed in 3/15 males and 1/15 females at 1300 mg/kg. Mice from the high dose replacement group were used for bone marrow collection, thus all of the high dose groups had 5 mice/sex available for bone marrow collection. Piloerection was seen in all males and all females treated with the test substance. Lethargy was noted in 2/5 males at 650 mg/kg and all males and all females at 1300 mg/kg. All males and all females treated with the control substances appeared normal following dose administration.

Based on bone marrow analysis/metaphase spreads, the following was observed:

- Reductions in the ratio of polychromatic erythrocytes to total erythrocytes (PCEs/ECs ratio) relative to the respective vehicle control groups of 23% and 13% were observed in the male and female 48 hour test substance-treated groups, respectively. With the exception of the female low dose group (reduction of 5%), no reductions in the PCEs/ECs ratio were observed in the 24 hour test substance-treated groups relative to the respective vehicle control groups, suggesting that the test substance did not inhibit erythropoiesis significantly.
- No statistically significant increase in the incidence of micronucleated polychromatic erythrocytes in test substance-treated groups relative to the respective vehicle control groups was observed in male or female mice at 24 or 48 hours after dose administration ($p > 0.05$, Kastenbaum-Bowman Tables).
- No statistically significant increase in the incidence of structural or numerical chromosome aberrations in test substance-treated groups relative to the respective vehicle control groups was observed in male or female mice at 24 or 48 hours after dose administration ($p > 0.05$, Fisher's Exact Test).

- Dose-dependant reductions in the mitotic index relative to the vehicle control groups of up to 43.2% and 53.4% were observed in the male and female 24 hour test substance-treated groups, respectively. Reductions in the mitotic index relative to the vehicle control groups of up to 45.2% and 29.3% were observed in the male and female 48 hour test substance-treated groups, respectively.
- CP, the positive control, induced a statistically significant increase in the incidence of micronucleated PCEs ($p \leq 0.05$, Kastenbaum-Bowman Tables) and a statistically significant increase in the incidence of structural chromosome aberrations ($p \leq 0.05$, Fisher's Exact Test) in both male and female mice. The number of micronucleated PCEs and chromosome aberrations in the vehicle control groups did not exceed the historical vehicle control range. Based upon this, all criteria for a valid test were met as specified in the protocol.

Under the conditions described in this report, a single oral administration of H-28072 at doses up to and including 1300 mg/kg (the maximum tolerated dose), did not induce a significant increase in the incidence of micronucleated polychromatic erythrocytes or structural or numerical chromosome aberrations in bone marrow of male and female ICR mice. Based upon this, H-28072 was concluded to be negative for induction of chromosome aberrations in bone marrow cells and micronucleated polychromatic erythrocytes in bone marrow and therefore was concluded to have no genotoxic/clastogenic potential.

2.0 PURPOSE

The purpose of this study was to evaluate the clastogenic potential of the test substance as measured by its ability to induce chromosome aberrations in bone marrow cells and micronucleated polychromatic erythrocytes in mouse bone marrow.

This study was conducted in compliance with the testing guidelines:

OECD Guideline 474 (Genetic Toxicology: Mammalian Erythrocytes Micronucleus Test) and Guideline 475 (Mammalian Bone Marrow Chromosome Aberration Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998.

European Commission Directive 2000/32/EC of May 19, 2000, Annex 4C-B12. Mutagenicity – *In Vivo* Mammalian Erythrocyte Micronucleus Test. No. L 136.

U.S. Environmental Protection Agency (EPA), Health Effects test Guidelines OPPTS 870.5395, Mammalian Erythrocyte Micronucleus Test (August 5, 1998) and OPPTS 870.5385, Mammalian Bone Marrow Chromosome Aberration Test (August 5, 1998).

Historical negative (vehicle) and positive control data are presented in Appendix I. Copies of the study protocol and amendment are included in Appendix II.

3.0 CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

3.1 Characterization of Test Substance

The test substance, H-28072, was received by BioReliance on 16 May 2007 and was assigned the code number AC03GE. Upon receipt, the test substance was described as a colorless liquid and was stored at ambient temperature (15 to 30°C), protected from exposure to light.

The Sponsor has indicated that the identity, strength, purity, composition or other characteristics of the test substance have been determined. A copy of the Certificate of Analysis is included in Appendix III. The initial Sponsor-reported purity was 84.5% active ingredient, but the Certificate of Analysis that was issued after the experimental termination of the study, reported a purity of 82.6%. In consultation with the Sponsor, all doses and concentrations in this report represent the nominal values based on the initial Sponsor-reported purity (84.5%). Since the Certificate of Analysis recommended a reanalysis interval of 1 year from the last analysis (19 July 2007), the test substance was considered stable for the purpose of this study.

3.2 Characterization of Control Substances

Sterile water (CAS No.: 7732-18-5; Lot No.: 49-526-DK; Expiration Date: 01 January 2010) obtained from Hospira was used as the vehicle control substance. Cyclophosphamide monohydrate (CP, CAS No.: 6055-19-2; Lot No.: 036K1225; Expiration Date: 31 March 2009) was obtained from Sigma-Aldrich Company and was dissolved in sterile water at a concentration of 5 mg/mL for use as the positive control substance.

The vehicle and positive control substances have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of the control substances and their mixtures was demonstrated by acceptable results that met the criteria for a valid test.

3.3 Preparation of Test Substance Dose Formulations

The test substance was prepared fresh for each phase of the study just prior to dose administration. The stock test substance formulation for the pilot toxicity study and all formulations for the toxicity and definitive studies were prepared as follows:

1. The test substance (adjusted for test substance purity using a correction factor of 1.18) was weighed into containers of appropriate size.

2. The vehicle was added to the test substance until the final batch volume was achieved.

3. Each formulation was vortexed for several minutes until clear, colorless solutions formed (32.5, 65, 120, 130, 140, 160, 180 and 200 mg/mL).

In the pilot toxicity study, concentrations of 0.1, 1, 10 and 100 mg/mL were prepared by serial dilution of the 200 mg/mL stock formulation with the vehicle. The dilutions were vortexed for several minutes resulting in clear, colorless solutions (0.1, 1, 10 and 100 mg/mL).

The initial Sponsor-reported purity for H-28072 was 84.5% active ingredient. A correction factor of 1.18 was used for preparation of dose formulations. However, the Certificate of Analysis, issued after the experimental termination of the study, reported a purity of 82.6%. Using an appropriate correction factor of 1.21, the recalculated doses achieved in this study did not differ from nominal significantly. Since the differences were considered negligible, it was concluded that this event did not impact the validity of the study conclusions.

4.0 MATERIALS AND METHODS

4.1 Test System

ICR mice were obtained from Harlan, Frederick, MD and were received on 12 June 2007 (pilot toxicity study), 26 June 2007 (toxicity study) and on 03 July 2007 (definitive micronucleus study). At the time of dose administration for each phase of the study, the mice were approximately 6 to 8 weeks old. Mouse body weights recorded at randomization were within the following ranges:

Pilot Toxicity Study:	Males: 27.5 – 30.4 g Females: 23.9 – 28.1 g
Toxicity Study:	Males: 26.8 – 29.6 g Females: 25.1 – 28.9 g
Definitive Micronucleus Study:	Males: 28.6 – 33.9 g Females: 25.0 – 30.5 g

4.2 Animal Receipt and Quarantine

Virus antibody-free (VAF) mice were obtained from a supplier that monitored mice for evidence of ectoparasites, endoparasites, pathogenic bacteria, mycoplasmas, and appropriate murine viruses and were quarantined (acclimatized) for no less than 5 days after receipt. At BioReliance, mice were observed each day for signs of illness and other conditions of poor health. All mice were judged to be healthy prior to utilization in the study.

4.3 Animal Welfare Provisions and Animal Care

This study is not duplicative or unnecessary. The number of mice and the procedures and experimental design used for this study have been reviewed and were approved by the BioReliance Institutional Animal Care and Use Committee #8, #9 and #10. All procedures involving mice performed at BioReliance follow the specifications recommended in *The Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington, D.C., 1996).

The mice were housed in an AAALAC-accredited facility with a controlled environment of 72 ± 3°F temperature, 50 ± 20% relative humidity, and a 12 hour light/dark cycle. Mice of the same sex were housed up to five per rodent Micro-Barrier cage. Cages were placed on the racks equipped with Micro-VENT full ventilation, HEPA filtered system. The purpose of this system is to supply uninterrupted positive air to each individual rodent Micro-Barrier cage and to capture the effluent air from each cage and re-filter the air (HEPA) prior to introducing the air back into the room. Heat-treated hardwood chips were used for bedding. Mice had free access to tap water and a certified laboratory rodent chow (Harlan 2018C Certified Global Rodent Diet), which has been analyzed for environmental contaminants. There were no contaminants in the feed that were considered to have influenced the results of the study. The water used in the study met USEPA drinking water standards and was monitored at least annually for levels of organophosphorus pesticides, metals, coliform bacteria, and other contaminants. Results of feed, water and bedding analyses are on file with BioReliance.

4.4 Pilot Toxicity Study

In the pilot toxicity study, mice were randomly assigned to one group of five male and five female mice each and to four groups of two males each. Each mouse was given a sequential number and identified by an ear tag. All mice were weighed immediately prior to dose administration and the administered volume was based on individual body weight. Five male and five female mice were exposed to H-28072 at 2000 mg/kg and two male mice each were exposed to H-28072 at 1, 10, 100 or 1000 mg/kg. The test substance dose formulations were administered at a volume of 10 mL/kg by a single oral gavage. Mice were observed after dose administration and daily thereafter for 3 days for clinical signs of toxicity. Body weights were recorded before dose administration on Study Day 0 and on Study Days 1 and 3. Following the last observation on Study Day 3, animals were euthanized by exposure to CO_2 and discarded without further examination.

4.5 Toxicity Study

In the toxicity study, mice were randomly assigned to four groups of five male and five female mice each. Each mouse was given a sequential number and identified by an ear tag. All mice in the experimental groups were weighed immediately prior to dose administration and the administered volume was based on individual body weight. The mice were exposed to H-28072 at 1200, 1400, 1600 or 1800 mg/kg. The test substance dosing formulations were administered at a volume of 10 mL/kg by a single oral gavage. Mice were observed after dose administration and daily thereafter for 3 days for clinical signs of toxicity. Body weights were recorded before dose administration on Study Day 0 and on Study Days 1 and 3. Following the last observation on Study Day 3, animals were euthanized by exposure to CO_2 and discarded without further examination.

4.6 Micronucleus and Chromosome Aberration Assay

The micronucleus and chromosome aberration assay was conducted using established and validated procedures (Heddle, J.A, 1973; Heddle, J.A., *et al*., 1983; Hayashi, M., *et al*., 1994; Mavournin, K., *et al*., 1990). The mice were assigned to seven experimental groups of five males and five females each according to a computer-generated program, which is based on distribution according to body weight. An additional group of five males and five females was designated as a replacement group to be used in the event of mortality at the high dose. Each mouse was given a sequential number and identified by an ear tag. The study design was as follows:

Treatment (10 mL/kg)	Number of Mice/Sex Dosed	Number of Mice/Sex Used for Bone Marrow Collection at	
		24 hrs post-dose	48 hrs post-dose
Vehicle Control: Sterile water	10	5	5
Test Substance: H-28072			
Low dose (325 mg/kg)	5	5	0
Mid dose (650 mg/kg)	5	5	0
High dose (1300 mL/kg)	15*	5	5
Positive Control: CP (50 mg/kg)	5	5	0

*Including 5 replacement mice/sex to ensure the availability of five mice for micronucleus analysis.

4.7 Dose Administration (Definitive Study)

In the definitive study, mice were exposed to H-28072 at 325, 650 or 1300 mg/kg, the vehicle control or the positive control substance. All dose formulations were administered by a single oral gavage at a dose volume of 10 mL/kg. The oral route of administration has been routinely used, validated and is widely-accepted for use in the mammalian bone marrow erythrocyte micronucleus assay. All mice in the experimental and control groups were weighed immediately before dose administration, and the administered volume was based on individual body weight. Mice were observed after dose administration and throughout the course of the study for clinical signs of toxicity.

Colchicine (CAS number 64-86-8, Lot number 086K1576), used to arrest cells in metaphase, was administered intraperitoneally at a dose volume of 4 mL/kg to all mice two to four hours prior to

scheduled sacrifice/bone marrow collection time. Colchicine was obtained from Sigma-Aldrich Company and was dissolved in Hanks' Balanced Salt Solution (Gibco, Lot number 1391329) at a concentration of 0.5 mg/mL.

4.8 Bone Marrow Collection and Slide Preparation

4.8.1 Collection of Bone Marrow Metaphase Cells for Chromosome Aberration Analysis

At the scheduled bone marrow collection time, five mice per sex per treatment were euthanized by CO_2 asphyxiation. Immediately following sacrifice, one femur was distally exposed, cut just above the knee, and the bone marrow was aspirated into a syringe containing cold Hank's Balanced Salt Solution (HBSS). The bone marrow cells were transferred to a labeled centrifuge tube containing 5 mL HBSS. Tubes were identified by study, group and animal numbers. The cells were mixed well and maintained in an ice bath until the bone marrow cells had been collected from all the animals.

4.8.2 Cell Fixation and Slide Preparation

The cells for metaphase analysis were prepared as follows:

1. The tubes were centrifuged at approximately 100 x g for 5-10 minutes.
2. The supernatant fluid was discarded, the cell pellet was dislodged and 5 mL of pre-warmed (~ 37°C) 0.065 M KCl was added to each tube.
3. The cells were resuspended and any large fat deposits were removed.
4. The cells were incubated at 37 ± 1°C for 10 minutes in a water bath.
5. Following incubation, the cells were mixed well and five drops of freshly prepared fixative (methanol:acetic acid, 3:1 v/v) were added to each tube.
6. The cells were collected by centrifugation and resuspended in two consecutive changes of fixative.
7. The tubes were capped and stored overnight or longer at approximately 2-8°C until the time of slide preparation.

For slide preparation, the cells were centrifuged at approximately 100 x g for 5-10 minutes, the supernatant fluid was decanted, and the cells were resuspended to opalescence in about 1 mL of fresh fixative. Two to four drops of cell suspension were dropped near the frosted end of a wet, cold glass slide and allowed to spread out to the opposite end of the slides and air dry. Two slides were prepared from each animal. The dry slides were stained with 5% Giemsa stain and permanently mounted.

4.8.3 Bone Marrow Collection and Slide Preparation for Micronucleus Analysis

For the micronucleus analysis, the smears were prepared as follows:

1. Immediately following sacrifice, the other femur was exposed, cut just above the knee, and the bone marrow was aspirated into a syringe containing fetal bovine serum.
2. The bone marrow cells were transferred to a labeled centrifuge tube containing approximately 1 mL fetal bovine serum.

3. The bone marrow cells were pelleted by centrifugation at approximately 100 x g for five minutes and the supernatant was drawn off, leaving a small amount of serum with the remaining cell pellet.
4. The cells were resuspended and a small drop of bone marrow suspension was spread onto a clean glass slide. Two slides were prepared from each mouse.
5. The slides were fixed in methanol, stained with May-Gruenwald-Giemsa and permanently mounted.

4.9 Scoring

4.9.1 Scoring for Chromosome Aberrations

Stained slides were coded using a random number table by an individual not involved with the scoring process and were scored without regard to treatment group. Cells were examined under the light microscope and oil immersion (1000X). A minimum of 100 metaphase spreads containing 40 (2n) centromeres were examined from each animal and scored for chromatid-type and chromosome-type aberrations. In the case that the high numbers of aberration, i.e. >10%, were observed during scoring, fewer cells (metaphase spreads) were scored.

The evaluation (scoring) of the metaphase spreads included:

- **Chromatid-type** aberrations include: chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials and complex rearrangements.
- **Chromosome-type** aberrations include chromosome breaks and exchange figures such as dicentrics and rings.
- **Fragments** (chromatid or acentric) observed in the absence of any exchange figure were scored as a break (chromatid or chromosome).
- **Fragments** observed with an exchange figure were not scored as an aberration but were considered part of the incomplete exchange.
- **Pulverized chromosome(s)**, pulverized cells and severely damaged cells (>10 aberrations) were also recorded.
- **Chromatid and isochromatid gaps** were recorded but not included in the analysis.
- **The XY coordinates** for each cell with structural aberrations were recorded using a calibrated microscope stage.
- **The mitotic index,** as indication of cytotoxicity, was recorded as the percentage of cells in mitosis based upon 1000 cells counted per animal.
- **Number of numerically damaged cells** (polyploid and endoreduplicated cells) was recorded per 100 metaphase cells per animal (for total of 500 cells per group).

4.9.2 Scoring for Micronuclei

To control for bias, bone marrow slides were coded using a random number table by an individual not involved with the scoring process. Using a light microscope and a medium magnification (400X), an area of acceptable quality was selected such that the cells were well spread and stained. Using oil immersion (1000X), the following cell populations and cellular components were evaluated and enumerated:

- **Polychromatic erythrocytes (PCEs)**
  PCEs stain bluish. PCEs are young erythrocytes (early stage of erythropoiesis) and are the target cells for evaluation of the test substance clastogenicity. Two-thousand PCEs per mouse were screened (scored) for the presence of micronuclei resulting in evaluation of a total of 10,000 PCEs per each treatment group.
- **Normochromatic erythrocytes (NCEs)**
  NCEs stain pink (redish). NCEs are mature erythrocytes (red blood cells) and are the final cell population formed during erythropoiesis. The number of NCEs and micronucleated NCEs (MNCEs) in the field of 1000 total erythrocytes (ECs) was determined for each animal in order to determine the proportion of polychromatic erythrocytes to total erythrocytes (PCEs/ECs). The incidence of MNCEs per 2000 PCEs was enumerated for each animal, but the results were not presented in this report or used in analysis of the test substance clastogenic response since the primary target cells are the PCEs.
- **Micronuclei (M)**
  Micronuclei are round, darkly-staining nuclear (chromosome) fragments with a sharp contour and diameters usually from 1/20 to 1/5 of an erythrocyte. Micronuclei may occur in PCEs (MPCEs) or NCEs (MNCEs).
- The proportion of polychromatic erythrocytes to total erythrocytes was also recorded per 1000 erythrocytes per each animal (PCEs/ECs ratio).

4.10 Evaluation of Test Results

4.10.1 Chromosome Aberrations Assay

The mitotic index and the total number and types of aberrations found in each animal are presented. Gaps are presented in the data but not included in the total percentage of cells with one or more aberrations or in the average number of aberrations per cell. The percentage of aberrant cells in the total population of cells scored was calculated for each treatment group. The severity of damage within the cells was reported as the average number of aberrations per cell for each treatment dose. Male and female animals were analyzed separately. The Fisher's exact test was used for pairwise comparisons of the number of aberrant cells between each treatment and the vehicle (vehicle) control group.

The conclusion of the study was based on sound scientific judgment. As a guide to interpretation of the data, the following was considered:

- The test substance would have been considered to induce a positive response in the chromosome aberration assay if the number of aberrant cells was significantly increased in a

dose responsive manner relative to the vehicle control ($p \leq 0.05$, Fisher's Exact Test) at any sampling time.

- Values that were statistically significant but did not exceed the range of historical vehicle controls (Appendix I) would have been judged as not biologically relevant or significant.
- The test substance was judged negative if no statistically significant increase in the number of aberrant cells above the concurrent vehicle control values and no evidence of dose response were observed at any sampling time.

4.10.2 Micronucleus Assay

The incidence of micronucleated polychromatic erythrocytes per 2000 PCEs for each mouse and per 10,000 PCEs for each treatment group was determined. Statistical significance was determined using the Kastenbaum-Bowman tables which are based on the binomial distribution (Kastenbaum and Bowman, 1970). All analyses were performed separately for each sex and sampling time.

In order to quantify the proliferation state of the bone marrow as an indicator of bone marrow toxicity, the proportion of polychromatic erythrocytes to total erythrocytes was determined for each mouse and treatment group (PCEs/ECs ratio). The proportion of polychromatic erythrocytes to total erythrocytes in test substance-treated animals should not be less than 20% of the control value.

All conclusions were based on a scientific judgment. As a guide to interpretation of the data, the following was considered:

- The test substance would have been considered to induce a positive response in the micronucleus assay if a dose-responsive increase in the incidence of micronucleated polychromatic erythrocytes was observed and one or more doses were statistically elevated relative to the vehicle control ($p \leq 0.05$, Kastenbaum-Bowman Tables) at any sampling time.

- Values that were statistically significant but did not exceed the range of historical vehicle controls (Appendix I) would have been judged as not biologically significant/relevant.
- The test substance was judged negative if no statistically significant increase in the incidence of micronucleated polychromatic erythrocytes above the concurrent vehicle control values and no evidence of dose response were observed at any sampling time.

4.11 Criteria for a Valid Test

4.11.1 Chromosome Aberration Assay

The percentage of cells in the vehicle control group demonstrating aberrations of any type, other than gaps, must not exceed the historical control range. The percentage of cells with aberrations in the positive control group must be statistically increased above that in the vehicle control group ($p \leq 0.05$, Fisher's Exact Test).

4.11.2 Micronucleus Assay

The mean incidence of micronucleated polychromatic erythrocytes in the vehicle control group must not exceed the historical vehicle control range. The incidence of micronucleated polychromatic erythrocytes in the positive control group must be significantly increased relative to the vehicle control group ($p \leq 0.05$, Kastenbaum-Bowman Tables).

4.12 Automated Data Collection Systems

The primary computer applications used for the collection of data at BioReliance included: *LIMS Labware version 5, configured version 1.0.3, Oracle Version 11.5.8 (Oracle Corporation), Excel 2003 (Microsoft Corporation) and Kaye Lab Watch Monitoring System Version 12.0 (Kaye GE).*

4.13 Records and Archives

All raw data, the protocol and all reports, generated by BioReliance, will be maintained according to Standard Operating Procedure OPQP3040 by the BioReliance QA unit headquartered at: BioReliance, 14920 Broschart Rd., Rockville, MD 20850. Per this SOP, paper records will be retained for at least three years after which time the Sponsor will be contacted for a decision as to the final disposition of the materials. All study materials returned to the Sponsor or destroyed will first be copied onto electronic media and the electronic copy will be maintained in the BioReliance archives for a minimum of 10 years.

Raw data, the protocol and reports generated at facilities other than BioReliance will be archived per the contractual arrangements between that facility and the Sponsor.

Unless alternate arrangements are made to the contrary, the testing facility at BioReliance will not retain residual test substance or the slides from the study. They will be disposed after the issuance of the final report.

4.14 Deviations

No known deviations from the protocol or assay method SOPs occurred during the conduct of this study.

5.0 RESULTS AND DISCUSSION

5.1 Pilot Toxicity Study

In the pilot toxicity study, H-28072 was administered to two male mice each at 1, 10, 100 or 1000 mg/kg and to five male and five female mice at 2000 mg/kg. Mortality, clinical signs and body weight data are reported in Tables 8.0-1 and 8.0-2. Mortality was observed in 4/5 males and 4/5 females at 2000 mg/kg. Piloerection was seen in 1/2 males at 1 mg/kg, all males at doses ≥ 10 mg/kg and all females at 2000 mg/kg. Lethargy and cool to the touch were noted in all males at 1000 and 2000 mg/kg and all females at 2000 mg/kg. No appreciable changes occurred in the mean

group animal body weights of males at doses ≤ 100 mg/kg. Appreciable reductions in the mean group animal body weights of up to 10.9% and 8.6% occurred in males at 1000 and 2000 mg/kg, respectively, and of up to 12.6% in females at 2000 mg/kg. In order to further assess toxicity of the test substance, a toxicity study was performed.

5.2 Toxicity Study

In the toxicity study, H-28072 was administered to male and female mice (5 mice/sex/group) at 1200, 1400, 1600 or 1800 mg/kg. Mortality, clinical signs and body weight data are reported in Tables 8.0-3 and 8.0-4. Mortality was observed in 1/5 males at 1400 mg/kg, 2/5 males and 1/5 females at 1600 mg/kg and 3/5 males and 2/5 females at 1800 mg/kg. Lethargy and piloerection were seen in all males and all females at all doses. No appreciable changes in the mean group animal body weights of males or females occurred at any of the doses. Based upon these results, the high dose for the definitive micronucleus study was set at 1300 mg/kg, which was estimated to be the maximum tolerated dose.

5.3 Definitive Study

In the definitive study, male and female mice were exposed to H-28072 at 325, 650 or 1300 mg/kg, the vehicle or positive control substance. Mortality and clinical signs are presented in Table 8.0-5. Mortality was observed in 3/15 males and 1/15 females at 1300 mg/kg. Mice from the high dose replacement group were used for bone marrow collection, thus all of the high dose groups had 5 mice/sex available for bone marrow collection. Piloerection was seen in all males and all females treated with the test substance. Lethargy was noted in 2/5 males at 650 mg/kg and all males and all females at 1300 mg/kg. All males and all females treated with the control substances appeared normal following dose administration.

5.3.1 Chromosome Aberration Assay

Bone marrow cells arrested in metaphase and collected 24 or 48 hours after treatment were examined microscopically for structural and numerical chromosome aberrations. The percentage of damaged cells in the total population of cells scored and the number of aberrations per cell for 24 and 48 hour treatment groups are presented in Tables 8.0-6 (summary table). The individual animal data for the mitotic index and the number and types of aberrations in the 24 and 48 hour treatment groups are presented in Tables 8.0-7 and 8.0-8, respectively.

Based on the analysis of metaphase spreads, the following was observed:

- No statistically significant increase in the incidence of structural or numerical chromosome aberrations in test substance-treated groups relative to the respective vehicle control groups was observed in male or female mice at 24 or 48 hours after dose administration ($p > 0.05$, Fisher's Exact Test).
- Dose-dependant reductions in the mitotic index relative to the vehicle control groups of up to 43.2% and 53.4% were observed in the male and female 24 hour test substance-treated groups, respectively. Reductions in the mitotic index relative to the vehicle control groups of up to 45.2% and 29.3% were observed in the male and female 48 hour test substance-treated groups, respectively.

- CP, the positive control, induced a statistically significant increase in the incidence of structural chromosome aberrations ($p \leq 0.05$, Fisher's Exact Test) in both male and female mice. The number of chromosome aberrations in the vehicle control groups did not exceed the historical vehicle control range. Based upon this, all criteria for a valid test were met as specified in the protocol.

5.3.2 Micronucleus Assay

Bone marrow cells [polychromatic erythrocytes (PCEs) collected 24 and 48 hours after treatment were examined microscopically for presence of micronuclei (MPCEs). The incidence of micronucleated polychromatic erythrocytes per 10,000 polychromatic erythrocytes scored (2000 PCEs/mouse) and the proportion of polychromatic erythrocytes per total erythrocytes are summarized and presented for each treatment group by sacrifice time in Table 8.0-9. Individual mouse data are presented in Tables 8.0-10 and 8.0-11.

Based on bone marrow analysis, the following was observed:

- Reductions in the ratio of polychromatic erythrocytes to total erythrocytes (PCEs/ECs ratio) relative to the respective vehicle control groups of 23% and 13% were observed in the male and female 48 hour test substance-treated groups, respectively. With the exception of the female low dose group (reduction of 5%), no reductions in the PCEs/ECs ratio were observed in the 24 hour test substance-treated groups relative to the respective vehicle control groups, suggesting that the test substance did not inhibit erythropoiesis significantly.
- No statistically significant increase in the incidence of micronucleated polychromatic erythrocytes in test substance-treated groups relative to the respective vehicle control groups was observed in male or female mice at 24 or 48 hours after dose administration ($p > 0.05$, Kastenbaum-Bowman Tables).
- No statistically significant increase in the incidence of structural or numerical chromosome aberrations in test substance-treated groups relative to the respective vehicle control groups was observed in male or female mice at 24 or 48 hours after dose administration ($p > 0.05$, Fisher's Exact Test).
- CP, the positive control, induced a statistically significant increase in the incidence of micronucleated PCEs ($p \leq 0.05$, Kastenbaum-Bowman Tables) in both male and female mice. The number of micronucleated PCEs in the vehicle control groups did not exceed the historical vehicle control range. Based upon this, all criteria for a valid test were met as specified in the protocol.

6.0 CONCLUSION

Under the conditions described in this report, a single oral administration of H-28072 at doses up to and including 1300 mg/kg (the maximum tolerated dose), did not induce a significant increase in the incidence of micronucleated polychromatic erythrocytes or structural or numerical chromosome aberrations in bone marrow of male and female ICR mice. Based upon this, H-28072 was concluded to be negative for induction of chromosome aberrations in bone marrow cells and micronucleated polychromatic erythrocytes in bone marrow and therefore was concluded to have no genotoxic/clastogenic potential.

7.0 REFERENCES

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8.0 DATA TABLES

Table 8.0-1: Pilot Toxicity Study - Clinical Signs Following a Single Oral Dose of H-28072 in ICR Mice

Treatment (10 mL/kg)	Observation	Number of Animals With Observed Signs/Total Number of Animals Dosed		Number of Animals Died/Total Number of Animals Dosed	
		Males	Females	Males	Females
H-28072 1 mg/kg	Piloerection	1/2	N/A	0/2	N/A
10 mg/kg	Piloerection	2/2	N/A	0/2	N/A
100 mg/kg	Piloerection	2/2	N/A	0/2	N/A
1000 mg/kg	Lethargy Piloerection Cool to the touch	2/2 2/2 2/2	N/A	0/2	N/A
2000 mg/kg	Lethargy Piloerection Cool to the touch	5/5 5/5 5/5	5/5 5/5 5/5	4/5	4/5

N/A = Female animals were not dosed as per study design.

Table 8.0-2: Pilot Toxicity Study - Body Weight and Mortality Data Following a Single Oral Dose of H-28072 in ICR Mice

		Group Mean Body Weights (g)			% Change[1]		
Treatment (10 mL/kg)	**Sex**	**Pretreatment**	**Day 1**	**Day 3**	**Day 1**	**Day 3**	**Mortality[2]**
H-28072							
1 mg/kg	M	30.4 ±0.6	30.9 ±1.1	31.1 ±1.3	1.6%	2.3%	0/2
10 mg/kg	M	29.4 ±1.6	30.3 ±1.3	31.2 ±0.9	3.1%	6.1%	0/2
100 mg/kg	M	30.0 ±1.0	29.8 ±0.6	30.4 ±0.6	-0.7%	1.3%	0/2
1000 mg/kg	M	30.2 ±0.8	26.9 ±1.1	29.6 ±2.4	-10.9%	-2.0%	0/2
2000 mg/kg	M	30.2 ±1.0	27.6 ±0.8	28.4 [3]SD	-8.6%	-6.0%	4/5
	F	27.7 ±1.6	24.2 ±0.8	24.4 [3]SD	-12.6%	-11.9%	4/5

[1]% Change = (Post-treatment weight - Pretreatment weight) x 100
Pretreatment weight

[2]Reported as number of mice found dead after dose administration/total number tested.

[3]SD = Standard deviation not available due to single surviving mouse.

Table 8.0-3: Toxicity Study - Clinical Signs Following a Single Oral Dose of H-28072 in ICR Mice

		Number of Animals With Observed Signs/Total Number of Animals Dosed		Number of Animals Died/Total Number of Animals Dosed	
Treatment (10 mL/kg)	**Observation**	**Males**	**Females**	**Males**	**Females**
H-28072 1200 mg/kg	Lethargy Piloerection	5/5 5/5	5/5 5/5	0/5	0/5
1400 mg/kg	Lethargy Piloerection	5/5 5/5	5/5 5/5	1/5	0/5
1600 mg/kg	Lethargy Piloerection	5/5 5/5	5/5 5/5	2/5	1/5
1800 mg/kg	Lethargy Piloerection	5/5 5/5	5/5 5/5	3/5	2/5

Table 8.0-4: Toxicity Study - Body Weight and Mortality Data Following a Single Oral Dose of H-28072 in ICR Mice

		Group Mean Body Weights (g)			% Change[1]		
Treatment (10 mL/kg)	**Sex**	**Pretreatment**	**Day 1**	**Day 3**	**Day 1**	**Day 3**	**Mortality[2]**
H-28072							
1200 mg/kg	M	30.0 ±0.9	29.9 ±1.6	31.2 ±1.6	-0.3%	4.0%	0/5
	F	27.6 ±2.5	28.5 ±2.6	28.7 ±1.8	3.3%	4.0%	0/5
1400 mg/kg	M	29.9 ±0.9	29.8 ±2.6	30.2 ±2.8	-0.3%	1.0%	1/5
	F	27.9 ±0.6	28.8 ±1.0	29.7 ±1.1	3.2%	6.5%	0/5
1600 mg/kg	M	29.8 ±1.1	29.8 ±1.1	30.3 ±1.4	0.0%	1.7%	2/5
	F	28.0 ±1.7	27.2 ±4.9	28.2 ±2.7	-2.9%	0.7%	1/5
1800 mg/kg	M	30.6 ±1.8	28.9 ±2.5	30.4 ±0.8	-5.6%	-0.7%	3/5
	F	28.2 ±0.6	27.2 ±2.2	29.7 ±0.3	-3.5%	5.3%	2/5

[1]% Change = $\frac{\text{(Post-treatment weight - Pretreatment weight) x 100}}{\text{Pretreatment weight}}$

[2]Reported as number of mice found dead after dose administration/total number tested.

Table 8.0-5: Micronucleus and Chromosome Aberration Assay - Clinical Signs Following a Single Oral Dose of H-28072 in ICR Mice

		Number of Animals With Observed Signs/Total Number of Animals Dosed		Number of Animals Died/Total Number of Animals Dosed	
Treatment (10 mL/kg)	**Observation**	**Males**	**Females**	**Males**	**Females**
Sterile water	Normal	10/10	10/10	0/10	0/10
H-28072 325 mg/kg	Piloerection	5/5	5/5	0/5	0/5
650 mg/kg	Lethargy Piloerection	2/5 5/5	0/5 5/5	0/5	0/5
1300 mg/kg	Lethargy Piloerection	15/15 15/15	15/15 15/15	3/15	1/15
Cyclophosphamide 50 mg/kg	Normal	5/5	5/5	0/5	0/5

Table 8.0-6: Summary of Chromosome Aberration Analysis Following a Single Oral Dose of H-28072 in ICR Mice

Treatment (10 mL/kg)	Sex	Time (Hr)	Number of Cells Evaluated	Mean Mitotic Index (%)†	Change From Control (%)	Cells with Aberrations Num[a].	Cells with Aberrations Struct.	Struct. Aberr.[1] (%)	Number of Aberrations Gaps	Number of Aberrations Breaks[2]	Number of Aberrations Exchs.	Number of Aberrations SDC[3]	Aberrations Per Cell[1] (mean ± SD)
Sterile water													
	M	24	500	8.8	--	0	2	0.4	0	2	0	0	0.004 ± 0.005
	F	24	500	8.8	--	0	0	0.0	0	0	0	0	0.000 ± 0.000
H-28072													
325 mg/kg	M	24	500	7.1	-19.3	0	1	0.2	0	1	0	0	0.002 ± 0.004
	F	24	500	7.0	-20.5	0	0	0.0	0	0	0	0	0.000 ± 0.000
650 mg/kg	M	24	500	5.9	-33.0	0	2	0.4	0	1	1	0	0.004 ± 0.005
	F	24	500	6.5	-26.1	0	0	0.0	0	0	0	0	0.000 ± 0.000
1300 mg/kg	M	24	500	5.0	-43.2	0	1	0.2	0	1	0	0	0.002 ± 0.004
	F	24	500	4.1	-53.4	0	1	0.2	0	1	0	0	0.002 ± 0.004
Cylcophosphamide													
50 mg/kg	M	24	250	2.9	-67.0	0	*44	18	0	96	1	50	0.588 ± 0.171
	F	24	250	3.1	-64.8	0	*56	22	0	111	2	30	0.572 ± 0.090
Sterile water													
	M	48	500	9.3	--	0	1	0.2	1	1	0	0	0.002 ± 0.004
	F	48	500	9.2	--	0	1	0.2	0	1	0	0	0.002 ± 0.004
H-28072													
1300 mg/kg	M	48	500	5.1	-45.2	0	0	0.0	0	0	0	0	0.000 ± 0.000
	F	48	500	6.5	-29.3	0	1	0.2	0	1	0	0	0.002 ± 0.004

*Statistically significant, $p \leq 0.05$ (Fisher's Exact Test).
[1]Excluding gaps.
[2]Includes chromatid and chromosome breaks and fragments.
[3]SDC=Cells having at least 10 aberrations of any type, including pulverized chromosomes or cells.
[4]Number of cells in mitosis per 1000 cells observed, expressed as a percentage (MI).
[5]Numerical aberrations include polyploid and endoduplicated cells.

Table 8.0-7: Induction of Chromosome Aberrations in Bone Marrow Collected 24 Hours Following a Single Oral Dose of H-28072 in ICR Mice

Treatment (10 mL/kg)	Sex	Animal Number	Cells Scored	MI (%)[1]	Number of Chromosome Aberrations: Structural Aberrations[2] — Chromatid Type: Gaps	Chromatid Type: Breaks	Chromatid Type: Exch.	Chromosome Type: Breaks	Chromosome Type: DIC	Chromosome Type: RG	SDC[3]	Numerical Aberrations[4]: Poly.	Endo.	Total
Sterile water														
	M	101	100	7.8	0	0	0	0	0	0	0	0	0	0
		102	100	8.8	0	1	0	0	0	0	0	0	0	0
		103	100	9.1	0	0	0	0	0	0	0	0	0	0
		104	100	9.6	0	0	0	0	0	0	0	0	0	0
		105	100	8.5	0	1	0	0	0	0	0	0	0	0
				MEAN MI= 8.8										
	F	106	100	10.1	0	0	0	0	0	0	0	0	0	0
		107	100	8.1	0	0	0	0	0	0	0	0	0	0
		108	100	9.4	0	0	0	0	0	0	0	0	0	0
		109	100	8.1	0	0	0	0	0	0	0	0	0	0
		110	100	8.5	0	0	0	0	0	0	0	0	0	0
				MEAN MI= 8.8										
H-28072														
325 mg/kg	M	111	100	7.0	0	0	0	0	0	0	0	0	0	0
		112	100	7.5	0	0	0	0	0	0	0	0	0	0
		113	100	7.0	0	1	0	0	0	0	0	0	0	0
		114	100	6.8	0	0	0	0	0	0	0	0	0	0
		115	100	7.2	0	0	0	0	0	0	0	0	0	0
				MEAN MI= 7.1										
	F	116	100	7.3	0	0	0	0	0	0	0	0	0	0
		117	100	6.8	0	0	0	0	0	0	0	0	0	0
		118	100	7.0	0	0	0	0	0	0	0	0	0	0
		119	100	7.4	0	0	0	0	0	0	0	0	0	0
		120	100	6.5	0	0	0	0	0	0	0	0	0	0
				MEAN MI= 7.0										
650 mg/kg	M	121	100	6.0	0	1	0	0	0	0	0	0	0	0
		122	100	6.2	0	0	0	0	0	0	0	0	0	0
		123	100	5.8	0	0	0	0	0	0	0	0	0	0
		124	100	5.6	0	0	0	0	0	0	0	0	0	0
		125	100	6.1	0	0	1	0	0	0	0	0	0	0
				MEAN MI= 5.9										

[1]Number of cells in mitosis per 1000 cells observed, expressed as a percentage (MI).
[2]Chromatid breaks includes chromatid and isochromatid breaks and fragments; Chromatid exchanges includes quadriradials, triradials and complex rearrangements; Chromosome breaks includes chromosome breaks and fragments; Dic represents dicentrics; Rg represents ring chromosomes.
[3]SDC=Cells having at least 10 aberrations of any type, including pulverized chromosomes or cells.
[4]Polyploid and endoduplicated cells.

Table 8.0-7 Continued: Induction of Chromosome Aberrations in Bone Marrow Collected 24 Hours Following a Single Oral Dose of H-28072 in ICR Mice

					Number of Chromosome Aberrations									
					Structural Aberrations[2]							Numerical Aberrations[4]		
Treatment		Animal	Cells		Chromatid Type			Chromosome Type						
(10 mL/kg)	Sex	Number	Scored	MI (%)[1]	Gaps	Breaks	Exch.	Breaks	DIC	RG	SDC[3]	Poly.	Endo.	Total
H-28072														
650 mg/kg	F	126	100	7.1	0	0	0	0	0	0	0	0	0	0
		127	100	5.9	0	0	0	0	0	0	0	0	0	0
		128	100	6.1	0	0	0	0	0	0	0	0	0	0
		129	100	6.2	0	0	0	0	0	0	0	0	0	0
		130	100	7.0	0	0	0	0	0	0	0	0	0	0
				MEAN MI= 6.5										
1300 mg/kg	M	131	100	5.2	0	0	0	0	0	0	0	0	0	0
		132	100	5.0	0	0	0	0	0	0	0	0	0	0
		133	100	4.8	0	0	0	0	0	0	0	0	0	0
		134	100	4.6	0	1	0	0	0	0	0	0	0	0
		135	100	5.2	0	0	0	0	0	0	0	0	0	0
				MEAN MI= 5.0										
	F	136	100	4.7	0	0	0	0	0	0	0	0	0	0
		137	100	4.8	0	0	0	0	0	0	0	0	0	0
		138	100	3.5	0	0	0	0	0	0	0	0	0	0
		139	100	3.7	0	1	0	0	0	0	0	0	0	0
		140	100	4.0	0	0	0	0	0	0	0	0	0	0
				MEAN MI= 4.1										
Cylcophosphamide														
50 mg/kg	M	141	50	4.0	0	34	1	0	0	0	0	0	0	0
		142	50	3.0	0	10	0	0	0	0	3	0	0	0
		143	50	2.0	0	15	0	0	0	0	1	0	0	0
		144	50	3.1	0	19	0	0	0	0	1	0	0	0
		145	50	2.5	0	18	0	0	0	0	0	0	0	0
				MEAN MI= 2.9										
	F	146	50	3.0	0	15	0	0	0	0	1	0	0	0
		147	50	3.0	0	18	0	0	0	0	1	0	0	0
		148	50	3.0	0	25	1	0	0	0	1	0	0	0
		149	50	3.5	0	29	0	0	0	0	0	0	0	0
		150	50	3.1	0	24	1	0	0	0	0	0	0	0
				MEAN MI= 3.1										

[1]Number of cells in mitosis per 1000 cells observed, expressed as a percentage (MI).
[2]Chromatid breaks includes chromatid and isochromatid breaks and fragments; Chromatid exchanges includes quadriradials, triradials and complex rearrangements; Chromosome breaks includes chromosome breaks and fragments; Dic represents dicentrics; Rg represents ring chromosomes.
[3]SDC=Cells having at least 10 aberrations of any type, including pulverized chromosomes or cells.
[4]Polyploid and endoduplicated cells.

Table 8.0-8: Induction of Chromosome Aberrations in Bone Marrow Collected 48 Hours Following a Single Oral Dose of H-28072 in ICR Mice

					Number of Chromosome Aberrations									
					Structural Aberrations[2]							Numerical Aberrations[4]		
Treatment		Animal	Cells		Chromatid Type			Chromosome Type						
(10 mL/kg)	Sex	Number	Scored	MI (%)[1]	Gaps	Breaks	Exch.	Breaks	DIC	RG	SDC[3]	Poly.	Endo.	Total
Sterile water														
	M	151	100	9.0	0	1	0	0	0	0	0	0	0	0
		152	100	9.4	1	0	0	0	0	0	0	0	0	0
		153	100	9.0	0	0	0	0	0	0	0	0	0	0
		154	100	9.9	0	0	0	0	0	0	0	0	0	0
		155	100	9.4	0	0	0	0	0	0	0	0	0	0
				MEAN MI= 9.3										
	F	156	100	9.1	0	0	0	0	0	0	0	0	0	0
		157	100	8.1	0	0	0	0	0	0	0	0	0	0
		158	100	10.0	0	0	0	0	0	0	0	0	0	0
		159	100	9.9	0	1	0	0	0	0	0	0	0	0
		160	100	8.9	0	0	0	0	0	0	0	0	0	0
				MEAN MI= 9.2										
H-28072														
1300 mg/kg	M	161	100	5.0	0	0	0	0	0	0	0	0	0	0
		162	100	4.1	0	0	0	0	0	0	0	0	0	0
		163	100	4.4	0	0	0	0	0	0	0	0	0	0
		164	100	7.9	0	0	0	0	0	0	0	0	0	0
		172*	100	4.0	0	0	0	0	0	0	0	0	0	0
				MEAN MI= 5.1										
	F	166	100	6.8	0	0	0	0	0	0	0	0	0	0
		176*	100	6.1	0	1	0	0	0	0	0	0	0	0
		168	100	7.0	0	0	0	0	0	0	0	0	0	0
		169	100	5.5	0	0	0	0	0	0	0	0	0	0
		170	100	7.1	0	0	0	0	0	0	0	0	0	0
				MEAN MI= 6.5										

[1]Number of cells in mitosis per 1000 cells observed, expressed as a percentage (MI).
[2]Chromatid breaks includes chromatid and isochromatid breaks and fragments; Chromatid exchanges includes quadriradials, triradials and complex rearrangements; Chromosome breaks includes chromosome breaks and fragments; Dic represents dicentrics; Rg represents ring chromosomes.
[3]SDC=Cells having at least 10 aberrations of any type, including pulverized chromosomes or cells.
[4]Polyploid and endoduplicated cells.
*Replacement animals

Table 8.0-9: Summary of Bone Marrow Micronucleus Analysis Following a Single Oral Dose of H-28072 in ICR Mice

Treatment (10 mL/kg)	Sex	Time (hr)	Number of Animals	PCE/Total Erythrocytes (Mean +/- SD)	Change from Control (%)	Number MPCE/1000 PCE (Mean +/- SD)	Number of MPCE/PCE Scored
Sterile water	M	24	5	0.431 ± 0.03	---	0.3 ± 0.27	3 / 10000
	F	24	5	0.425 ± 0.08	---	0.3 ± 0.27	3 / 10000
H-28072							
325 mg/kg	M	24	5	0.473 ± 0.06	10	0.0 ± 0.00	0 / 10000
	F	24	5	0.404 ± 0.06	-5	0.1 ± 0.22	1 / 10000
650 mg/kg	M	24	5	0.463 ± 0.04	7	0.4 ± 0.42	4 / 10000
	F	24	5	0.441 ± 0.04	4	0.0 ± 0.00	0 / 10000
1300 mg/kg	M	24	5	0.506 ± 0.10	17	0.1 ± 0.22	1 / 10000
	F	24	5	0.439 ± 0.09	3	0.3 ± 0.27	3 / 10000
Cyclophosphamide							
50 mg/kg	M	24	5	0.384 ± 0.08	-11	11.0 ± 2.42	*110 / 10000
	F	24	5	0.342 ± 0.05	-20	9.8 ± 1.20	*98 / 10000
Sterile water	M	48	5	0.440 ± 0.09	---	0.2 ± 0.27	2 / 10000
	F	48	5	0.448 ± 0.10	---	0.0 ± 0.00	0 / 10000
H-28072							
1300 mg/kg	M	48	5	0.340 ± 0.06	-23	0.3 ± 0.27	3 / 10000
	F	48	5	0.390 ± 0.06	-13	0.1 ± 0.22	1 / 10000

*Statistically significant, $p \leq 0.05$ (Kastenbaum-Bowman Tables)

Table 8.0-10: Induction of Micronucleated Polychromatic Erythrocytes in Bone Marrow Collected 24 Hours Following a Single Oral Dose of H-28072 in ICR Mice

Treatment (10 mL/kg)	Sex	Animal Number	PCE/Total Erythrocytes	Micronucleated PCE (Number/PCE scored)
Sterile water	M	101	0.380	1 / 2000
		102	0.430	0 / 2000
		103	0.446	1 / 2000
		104	0.440	1 / 2000
		105	0.461	0 / 2000
	F	106	0.332	0 / 2000
		107	0.486	0 / 2000
		108	0.495	1 / 2000
		109	0.341	1 / 2000
		110	0.469	1 / 2000
H-28072				
325 mg/kg	M	111	0.480	0 / 2000
		112	0.550	0 / 2000
		113	0.460	0 / 2000
		114	0.389	0 / 2000
		115	0.487	0 / 2000
	F	116	0.320	0 / 2000
		117	0.410	0 / 2000
		118	0.440	0 / 2000
		119	0.368	0 / 2000
		120	0.480	1 / 2000
650 mg/kg	M	121	0.471	2 / 2000
		122	0.490	1 / 2000
		123	0.433	0 / 2000
		124	0.419	1 / 2000
		125	0.502	0 / 2000
	F	126	0.460	0 / 2000
		127	0.374	0 / 2000
		128	0.490	0 / 2000
		129	0.433	0 / 2000
		130	0.450	0 / 2000
1300 mg/kg	M	131	0.614	0 / 2000
		132	0.599	0 / 2000
		133	0.426	0 / 2000
		134	0.395	1 / 2000
		135	0.496	0 / 2000
	F	136	0.444	1 / 2000
		137	0.557	0 / 2000
		138	0.476	0 / 2000
		139	0.415	1 / 2000
		140	0.305	1 / 2000
Cyclophosphamide	M	141	0.293	23 / 2000
50 mg/kg		142	0.395	19 / 2000
		143	0.382	30 / 2000
		144	0.500	18 / 2000
		145	0.352	20 / 2000
	F	146	0.359	17 / 2000
		147	0.282	23 / 2000
		148	0.309	18 / 2000
		149	0.392	19 / 2000
		150	0.369	21 / 2000

Table 8.0-11: Induction of Micronucleated Polychromatic Erythrocytes in Bone Marrow Collected 48 Hours Following a Single Dose of in ICR Mice

Treatment (10 mL/kg)	Sex	Animal Number	PCE/Total Erythrocytes	Micronucleated PCE (Number/PCE scored)
Sterile water	M	151	0.342	1 / 2000
		152	0.474	0 / 2000
		153	0.350	0 / 2000
		154	0.472	0 / 2000
		155	0.564	1 / 2000
	F	156	0.290	0 / 2000
		157	0.532	0 / 2000
		158	0.420	0 / 2000
		159	0.460	0 / 2000
		160	0.540	0 / 2000
H-28072 1300 mg/kg	M	161	0.310	0 / 2000
		162	0.380	1 / 2000
		163	0.420	1 / 2000
		164	0.256	1 / 2000
		172*	0.336	0 / 2000
	F	166	0.480	0 / 2000
		176*	0.420	0 / 2000
		168	0.383	1 / 2000
		169	0.330	0 / 2000
		170	0.337	0 / 2000

*Replacement animals.

9.0 APPENDICES

9.1 Appendix I: Historical Control Data

Mouse Micronucleus Test Historical Control Data
2004-2006

Negative Control[1]

Parameter	Ratio of PCE/Total Erythrocytes		Number of MPCE/2000 PCE Scored/Animal		Number of MPCE/10000 PCE Scored/Group	
	Males	Females	Males	Females	Males	Females
Mean[3]	0.50	0.49	0.73	0.75	3.63	3.77
Standard Deviation	0.06	0.07	0.73	0.71	2.31	2.00
Range[4]	0.24 - 0.69	0.23 - 0.68	0 - 3	0 - 3	0 -12	0 - 9

Positive Control[2]

Parameter	Ratio of PCE/Total Erythrocytes		Number of MPCE/2000 PCE Scored/Animal		Number of MPCE/10000 PCE Scored/Group	
	Males	Females	Males	Females	Males	Females
Mean[3]	0.37	0.37	34.90	34.40	174.51	171.99
Standard Deviation	0.07	0.07	11.45	12.42	46.66	51.58
Range[4]	0.14 - 0.63	0.13 - 0.65	9 - 77	8 - 82	59 - 328	70 – 317

[1]Since no appreciable differences in the induction of MPCEs by different vehicles and solvents (test substance carriers) and different routes of administration were observed, this table contains data from carriers and routes of administration widely used during the conduct of contract studies in the period of 2004-2006 at BioReliance.
Vehicles: water, water soluble vehicles (methylcellulose, carboxymethylcellulose, dextrose), saline, corn oil and other vehicles.
Routes of administration: intraperitoneal (IP), intravenous (IV), oral gavage (PO), subcutaneous (SC).
Bone marrow collection time: 24 and 48 hours post-dose.

[2]Positive control substance: Cyclophosphamide monohydrate (CP); Doses: 40 to 50 mg/kg; Routes of administration: IV, IP, PO. Bone marrow collection time: 24 hours post-dose.

[3] Average of the PCE ratio observed out of 1000 erythrocytes scored per animal for the total number of animals used during 2004-2006; average of the number of MPCE per 2000 PCE for the total number of animals used in 2004-2006; average of number of MPCE/per group (containing 5 animals per group) for total number of groups used in 2004-2006.

[4] Minimum and maximum range of PCE ratio observed out of 1000 erythrocytes scored per animal; the minimum and maximum range of MPCE observed out of 2000 PCE for the total number of animals used in 2004-2006 and the minimum and maximum range of MPCE observed out of 10000 PCE for the total number of groups used in 2004-2006.

Rodent[5] Bone Marrow Metaphase Analysis

1996 - 2006

Negative Control[1]

	Aberrant Cells[2] (%)		Aberrations/Cell[3]	
Parameter	Males	Females	Males	Females
Mean	0.22%	0.16%	0.002	0.002
Standard Deviation	0.30%	0.22%	0.003	0.002
Range	0.00% - 1.20%	0.00% - 0.80%	0.000 - 0.012	0.000 - 0.008

Positive Control[4]

	Aberrant Cells[2] (%)		Aberrations/Cell[3]	
Parameter	Males	Females	Males	Females
Mean	18.3%	19.4%	1.201	1.200
Standard Deviation	10.0%	9.7%	0.880	0.926
Range	7.4% - 40.6%	7.0% – 38.2%	0.260 – 3.364	0.268 – 3.440

[1]Negative controls include all vehicles and all routes of administration.
[2]Excluding cells containing gaps only.
[3]Excluding gaps; severely damaged cells were counted as 10 aberrations per cell.
[4]Positive control animals were dosed 20-50 mg Cyclophosphamide/kg body weight.
[5]Data from both mice and rats.

9.2 Appendix II: Study Protocol and Amendment

QA Reviewed
No. 15-A2007
Init. 21 Jun 07
Date

PROTOCOL AMENDMENT 1

SPONSOR: **E.I. du Pont de Nemours and Company**

TEST ARTICLE I.D.: **DuPont Haskell Laboratory**

BIORELIANCE STUDY NO: **AC03GE.123108.BTL**

PROTOCOL TITLE: **H-28072: In Vivo Micronucleus and Chromosome Aberration Assay in Mouse Bone Marrow Cells**

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1. **LOCATION:** Page 2, add new section 3.5 Storage Temperature

   AMENDMENT: Storage temperature is "Ambient (15 to 30°C)."

   REASON FOR THE AMENDMENT: To add test article storage temperature to the protocol

2. **LOCATION:** Page 6 and 7

   AMENDMENT: Add Section number 7.9 to Bone Marrow Collection, correct section # "7.5" for Scoring for Micronuclei to section # "7.10" and section # "7.6" Scoring for Chromosome Aberration to section #"7.11".

   REASON FOR THE AMENDMENT: To correct the errors in section numbers.

3. **LOCATION:** Page 7 Section 7.11 Scoring for Chromosome Aberrations

   AMENDMENT: Add the following at the end of the section: The percent polyploid and endoreduplicated cells will be evaluated per 100 metaphase cells per animal.

   REASON FOR THE AMENDMENT: To add the collection numerical aberration data in the chromosome aberration scoring.

APPROVALS:

Ramadevi Gudi — BIORELIANCE STUDY DIRECTOR — 18 Jun 2007 — DATE

Lisa Lucero — BIORELIANCE STUDY MANAGEMENT — 19 June 2007 — DATE

E. Jean Dunn — SPONSOR REPRESENTATIVE — 29 JUN 2007 — DATE

QA Reviewed
AD for WP15Jun2007
AD 15 Jun 07
Init. Date

Received by RA/QA 12-Jun-2007

DuPont–23220

BioReliance Study Number: AC03GE.123108.BTL

H-28072: In Vivo Micronucleus and Chromosome Aberration Assay in Mouse Bone Marrow Cells

1.0 PURPOSE

The purpose of this study is to evaluate the clastogenic potential of the test substance as measured by its ability to induce micronucleated polychromatic erythrocytes and chromosome aberrations in mouse bone marrow cells.

2.0 SPONSOR

2.1	Name:	E.I. du Pont de Nemours and Company
2.2	Address:	DuPont Haskell Laboratory P.O. Box 50 1090 Elkton Road Newark, DE 19714-0050
2.3	Representative:	Maria Donner, Ph.D. Phone: 302-366-5251 Fax: 302-366-5207 E-mail: maria.donner@usa.dupont.com
2.4	Sponsor Project #:	23220
2.5	WR #:	17319
2.6	Haskell #:	H-28072
2.7	Service Code:	553/572

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1	Test Substance Name:	H-28072	
3.2	Test Substance I.D.:	H-28072	
3.2	Controls:	Vehicle:	Test substance vehicle (sterile water for injection)
		Positive:	Cyclophosphamide (CP)
3.3	Purity:	84.5%, an adjustment for purity or active ingredient will be made using a correction factor of 1.18	

3.4 Test Substance Retention Sample

The retention of a reserve sample of the test substance will be the responsibility of the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Toxicology Testing Facility
BioReliance

4.2 Address: 9630 Medical Center Drive
Rockville, MD 20850

4.3 Study Director:Ramadevi Gudi, Ph.D.
Phone: (301) 610-2169
Fax: (301) 738-2362
E-mail:rgudi@bioreliance.com

5.0 TEST SCHEDULE

5.1 Experimental Start Date: 18-Jun-2007

5.2 Experimental Termination Date: 30 July 2007

5.3 Draft Report Date: 14 August 2007

5.4 Final Report Date: 2 weeks after Sponsor approves draft

6.0 TEST SYSTEM

Closed-colony, random-bred rodents are acceptable models for mutagenicity studies. ICR mice were selected because of the availability of historical control data.

6.1 Source: Harlan, Frederick, MD or other alternate Harlan location,
Charles River Breeding Laboratories, Kingston, NY or Raleigh, NC or other approved alternates

6.2 Number of Animals:

Dose Range Finding Phase: 13 male mice and 5 female mice (pilot toxicity study);
20 animals/sex (toxicity study);
Additional animals may be used, if the maximum tolerated dose was not determined.

Definitive Micronucleus Study: 35 animals/sex; Additional animals may be used as replacement animals in the case of mortality at the highest dose.

The number of animals/sex/group and dose levels (3) are selected in an attempt to produce graded responses to the test article. The number of animals is sufficient to permit meaningful evaluation of the data taking in consideration anticipated inter-animal variability.

6.3 Age at the Time of Dose Administration: ~6-8 weeks

6.4 Body Weight Range at the Time of Randomization: Male: 25.5-37.0 grams
Female: 19.5-31.0 grams

6.5 Animal Receipt and Acclimation

Virus antibody-free (VAF) mice will be acclimated (quarantined) for no less than 5 days prior to dose administration. The animals will be observed each day for signs of illness and other general conditions of poor health. All animals will be judged to be healthy prior to utilization in the study.

6.6 Animal Welfare Provisions

This study is not duplicative or unnecessary. The number of animals, animal procedures and experimental design used for this study have been reviewed and were approved by the BioReliance Institutional Animal Care and Use Committee (Protocol #8, #9 and #10). All procedures involving animals performed at BioReliance follow the specifications recommended in *The Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996).

6.7 Animal Care

Animals will be housed in an AAALAC-accredited facility with a controlled environment of 50 ± 20% relative humidity and 72 ± 3°F with a 12 hour light/dark cycle. The animal rooms will be supplied with at least 10 changes of fresh HEPA-filtered air every hour.

Mice of the same sex will be housed up to five per rodent Micro-Barrier cage. Cages will be placed on the racks equipped with an automatic watering system and Micro-VENT full ventilation, HEPA filtered system. The purpose of this system is to supply uninterrupted positive air to each individual rodent Micro-Barrier cage and to capture the effluent air from each cage and re-filter the air (HEPA) prior to introducing the air back into the cage.

Heat-treated hardwood chips will be used for bedding to absorb liquids (P.J. Murphy Forest Products, Montville, NJ).

Animals will have free access to tap water, which meets U.S. EPA drinking water standards [water source is Washington Suburban Sanitary Commission (WSSC) Potomac Plant]. Drinking water is monitored at least annually for levels of specified microorganisms, pesticides, heavy metals, alkalinity and halogens.

A certified laboratory rodent chow (Harlan 2018C Certified Global Rodent Diet) will be provided *ad libitum*. The food is analyzed by the manufacturer for the concentrations of specified heavy metals, aflatoxin, chlorinated hydrocarbons, organophosphates and specified nutrients.

The results of bedding, food and water analyses are on file at BioReliance. There are no contaminants to the bedding, feed and water that are expected to interfere with the study.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The assay will be conducted according to established procedures (Heddle, 1973; Mavournin *et al.*, 1990; Hayashi *et al.*, 1994). Following the administration of three concentrations of test substance as well as positive and negative (vehicle) controls to male and female mice, bone marrow cells will be collected at 24 and 48 hours and examined for the presence of micronucleated polychromatic erythrocytes and chromosome aberrations. The clastogenic potential of the test substance will be measured by its ability to increase micronucleated polychromatic erythrocytes and chromosome aberrations in treated animals as compared to vehicle control animals. The study design will be as follows:

Treatment	Number of Animals/Sex to be Dosed and Used in Bone Marrow Collection at	
	24 Hrs post-dose	48 Hrs post-dose
Negative control: **Sterile water for injection**	5	5
Test Substance: **H-28072**		
Low Dose	5	-
Mid Dose	5	-
High Dose	5	5
Positive Control: **Cyclophosphamide** CP (50 mg/kg)	5	-

7.1 Solubility Determination

Sterile water for injection will be the test substance vehicle.

7.2 Dose Selection

7.2.1 Dose Range Finding Phase

Selection of doses for the definitive micronucleus and chromosome aberration assay will be based on the toxicity of the test article but will not exceed 2000 mg/kg (the highest regulatory required dose). In the absence of toxicity data, a pilot study will be performed at a dose of 2000 mg/kg using up to five male and five female mice. Three or more lower doses will be tested using two male mice each.

In the event of mortality at 2000 mg/kg or any other doses in the pilot study, an extensive toxicity study will be performed using up to four test article doses, each containing up to five male and five female mice.

Animals will be assigned to the experimental groups using a randomization procedure based on equalization of group mean body weights. At the time of randomization, the weight variation of animals will not exceed ±20% of the mean weight.

Mice will be observed after dose administration and each day thereafter for 3 days for clinical signs of toxicity. Body weights will be recorded prior to dose administration (Study Day 0) and on Study Days 1 and 3 (prior to euthanasia). Following observation period, all surviving animals will be euthanized by exposure to CO_2 and discarded without further examination.

7.3 Route and Frequency of Administration

Animals will be dosed by oral gavage. Oral gavage was selected to maximize delivery of the test substance to the target system. Oral gavage is an acceptable method for administration of test substance concentrations to laboratory animals. Animals will receive the test substance as a single administration.

7.4 Controls

7.4.1 Vehicle control

The solvent vehicle for the test substance will be used as the vehicle control.

7.4.2 Positive control

Cyclophosphamide monohydrate (CP, CAS number 6055-19-2) will be administered as the positive control at a dose of 30-60 mg/kg. CP will be administered by the same route as the test substance. CP will be used as a

positive control for the clastogenic activity. No positive control will be used of numerical aberrations (polyploidy and/or endoreduplications).

7.5 Animal Receipt and Quarantine

Virus antibody-free (VAF) mice will be quarantined for no less than 5 days prior to dose administration. The animals will be observed each working day for signs of illness, unusual food and water consumption, and other general conditions of poor health. All animals will be judged to be healthy prior to utilization in the study.

7.6 Animal Care

Animals will be housed in an AAALAC-accredited facility with a controlled environment of 50 ± 20% relative humidity and 72 ± 3°F with a 12 hour light/dark cycle. Mice of the same sex will be housed up to five per cage in plastic autoclavable cages. Heat-treated hardwood chips will be used for bedding. Animals will have free access to a certified laboratory rodent chow, which has been analyzed for environmental contaminants and to tap water.

7.7 Randomization

The animals will be assigned to seven groups of five males and five females each using a randomization procedure based on equalization of group mean body weights. At the time of randomization, the weight variation of animals will not exceed ±20% of the mean weight. Additional animals may be designated and dosed as replacement animals in the high dose group to be used in the event of mortality prior to the scheduled sacrifice. This will be done at the discretion of the Study Director after evaluation of the toxicity data. Each animal will be given a sequential number and identified by ear tag.

7.8 Dose Preparation and Administration

The test substance formulation, the negative control alone and the positive control (CP) will be given as a single administration. The volume of administration will be 10 mL/kg body weight. All mice in the experimental groups will be weighed and the dose volume will be based on individual body weight. Animals will be observed for clinical signs of toxicity following dose administration and during the course of the study.

Colchicine, used to arrest dividing cells at metaphase, will be administered intraperitoneally at 1 to 2 mg/kg to all mice two to four hours prior to sacrifice.

Bone Marrow Collection

Approximately two to four hours after injection of colchicine, the mice will be sacrificed by carbon dioxide asphyxiation. The positive control group will be

sacrificed 24 hours after dose administration. One femur will be exposed, cut just above the knee and the marrow aspirated into a syringe containing cold Hanks' balanced salt solution. The cells will be collected by centrifugation, resuspended in 5 ml warm hypotonic solution (0.065M KCl) and then incubated for approximately 10 min at 37°C to swell the cells. The cells will be collected by centrifugation, resuspended in two consecutive changes of fixative (methanol:acetic acid, 3:1 v/v), capped and stored overnight at approximately 2-8°C. If necessary, fixed cells may be stored in the refrigerator for a few days prior to slide preparation. To prepare slides, the cells will be collected by centrifugation, resuspended to opalescence in fresh fixative. Two to four drops of fixed cells will be dropped onto a wet slide and air-dried. Each slide will be identified by the study number and animal number. At least two slides will be prepared from each animal, air dried, stained with Giemsa stain and permanently mounted.

The second femur will be exposed, cut just above the knee and the bone marrow will be aspirated into a syringe containing fetal bovine serum. The bone marrow cells will be transferred to a capped centrifuge tube containing approximately 1 mL fetal bovine serum. The bone marrow cells will be pelleted by centrifugation and the supernatant will be drawn off, leaving a small amount of fetal bovine serum with the remaining cell pellet. The cells will be resuspended and a small drop of the bone marrow suspension will be spread onto a clean glass slide. Each slide will be identified by the study number and animal number. At least two slides will be prepared from each animal, air dried, fixed by dipping in methanol, stained with May-Gruenwald-Giemsa stain and permanently mounted.

7.5 Scoring for Micronuclei

Slides will be coded using a random number table by an individual not involved with the scoring process. Using medium magnification, an area of acceptable quality will be selected such that the cells are well spread and stained. Using oil immersion, 2000 polychromatic erythrocytes will be scored per animal for the presence of micronuclei. The number of micronucleated normocytes in the field of 2000 polychromatic erythrocytes will also be enumerated. The proportion of polychromatic erythrocytes to total erythrocytes will also be recorded per 1000 erythrocytes. The proportion of polychromatic erythrocytes to total erythrocytes in test substance-treated animals should not be less than 20% of the control value.

7.6 Scoring for Chromosome Aberrations

Slides will be coded using a random number table by an individual not involved with the scoring process. Metaphase cells will be examined under oil immersion without prior knowledge of treatment groups. Where possible, a minimum of 100 metaphase spreads containing 40 (2n) centromeres will be examined from each animal and scored for chromatid-type and chromosome-type aberrations. Fewer cells may be scored when high numbers of aberrations, i.e., >10%, are observed. Chromatid-type

aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure will be scored as a break (chromatid or chromosome). Fragments observed with an exchange figure will not be scored as an aberration but will be considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells (>10 aberrations) will also be recorded. Chromatid and isochromatid gaps will be recorded but not included in the analysis. The XY coordinates for each cell with structural aberrations will be recorded using a calibrated microscope stage. The mitotic index will be recorded as the percentage of cells in mitosis based upon 1000 cells counted.

7.12 Automated Data Collection Systems

The computer or electronic systems used for the collection or analysis of data at BioReliance will include but not be limited to the following:

LIMS Labware version 5, configured version 1.0.3; Oracle (Oracle Corporation), Excel 2003 (Microsoft Corporation) and Kaye Lab Watch Monitoring system (Kaye GE).

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The number of micronucleated polychromatic erythrocytes in the negative control group must not exceed the historical negative (vehicle) control range. The incidence of micronucleated polychromatic erythrocytes in the positive control group must be significantly increased relative to the vehicle (negative) control ($p \leq 0.05$, Kastenbaum-Bowman Tables).

The percentage of cells in the negative control group demonstrating aberrations of any type, other than gaps, must not exceed historical control range. The percentage of aberrant cells in the positive control group must be statistically increased above that in the negative control ($p \leq 0.05$, Fisher's exact test).

Should one of these criteria not be met, the Sponsor will be contacted and possibility of the repeating the affected portion of the study will be discussed.

9.0 EVALUATION OF TEST RESULTS

The incidence of micronucleated polychromatic erythrocytes per 2000 polychromatic erythrocytes will be presented for each animal and treatment group. Statistical significance will be determined using the Kastenbaum-Bowman tables which are based on the binomial distribution. In order to quantify the test substance effect on

erythropoiesis, as an indicator of bone marrow toxicity, the proportion of polychromatic erythrocytes to total erythrocytes will be presented for each animal and treatment group.

The mitotic index and the total number and types of aberrations found in each animal will be presented. Gaps will be presented in the data but not included in the total number of cells with chromosome aberrations or in the average number of chromosome aberrations per cell. The percentage of aberrant cells in the total population of cells scored will be calculated for each treatment group. The severity of damage within the cells will be reported as the average number of aberrations per cell for each treatment dose. The Fisher's exact test will be used for pairwise comparisons of the number of aberrant cells between each treatment and negative control group. The Cochran-Armitage trend test using the number of aberrant cells per total cells scored will be performed to test for evidence of a dose response (if any).

All conclusions will be based on sound scientific judgement; however as a guide to interpretation of the data, the test substance will be considered to induce a positive response if the number of aberrant cells is significantly increased in a dose responsive manner relative to the negative control. However, values that are statistically significant but do not exceed the range of historical negative or vehicle controls may be judged as not biologically significant. If a single treatment group is significantly elevated at one sacrifice time with no evidence of a dose-response or if there is evidence of a dose response with no evidence of a significant increase in any treated group relative to the control group, the Sponsor will be contacted. The test substance will be judged negative if no statistically significant increase in number of aberrant cells above the concurrent negative control control values and no evidence of dose response are observed at any sampling time.

The test substance will be considered to induce a positive response if a dose-responsive increase in the incidence of micronucleated polychromatic erythrocytes is observed and one or more doses are statistically elevated relative to the negative control control ($p \leq 0.05$, Kastenbaum-Bowman Tables) at any sampling time. However, values that are statistically significant but do not exceed the range of historical negative or negative control controls may be judged as not biologically significant. If a single treatment group is significantly elevated at one sacrifice time with no evidence of a dose-response or if there is evidence of a dose response with no evidence of a significant increase in any treated group relative to the control group, the Sponsor will be contacted. The test substance will be judged negative if no statistically significant increase in micronucleated polychromatic erythrocytes above the concurrent negative control values and no evidence of dose response are observed at any sampling time.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data. The report will include:

- Test substance: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of the test substance, if known.

- Solvent/Vehicle: justification for choice of negative control; solubility and stability of the test substance in the solvent/vehicle, if known.

- Test animals: species and strain of animals used; number, age and sex of animals; source, housing conditions, diet, etc.

- Test conditions: positive and negative (vehicle/solvent) control data; data from range-finding study, if conducted; rationale for dose level selection; details of test substance preparation; details of the administration of test substance; rationale for route of administration; methods for verifying that the test substance reached the general circulation or target tissue, if applicable; details of food and water quality; description of treatment and sampling schedules; method of slide preparation; methods for measurement of toxicity; criteria for scoring micronucleated immature erythrocytes; number of cells analyzed per animal; criteria for considering study as positive, negative or equivocal, criteria for scoring aberrations; number of cells analysed per animal; criteria for considering study as positive, negative or equivocal.

- Results: signs of toxicity; proportion of polychromatic erythrocytes among total erythrocytes; number of micronucleated polychromatic erythrocytes per animal; mean±standard deviation of micronucleated polychromatic erythrocytes per group; dose-response relationship, where possible; statistical analyses; concurrent negative control data; historical negative control data with ranges, means and standard deviations; concurrent positive control data. signs of toxicity; mitotic index; type and number of aberrations per animal; total number of aberrations per group; number of cells with aberrations per group; changes in ploidy, if seen; dose-response relationship, where possible; statistical analyses; concurrent negative control data; historical negative control data with ranges, means and standard deviations; concurrent positive control data.

- Discussion of results.

- Conclusion.

- Appendices: Historical Control Data (negative and positive controls with ranges, means and standard deviations), copy of protocol and any amendment, and, if provided by the Sponsor, copies of the analyses that characterized the test substance, its stability and the stability and strength of the dosing preparations.

- Statement of Compliance

- Quality Assurance Statement

If an electronic copy of the protocol, the report or another study document is provided by BioReliance, the executed paper document is considered the official master document. If there is a discrepancy between an electronic copy and the corresponding master document, the master document will be considered the official document.

11.0 RECORDS AND ARCHIVES

All raw data, the protocol and all reports, generated by BioReliance, will be maintained according to Standard Operating Procedure OPQP3040 by the BioReliance QRA unit headquartered at: BioReliance, 14920 Broschart Road, Rockville, MD 20850. Per this SOP, paper records will be retained for at least three years after which time the Sponsor will be contacted for a decision as to the final disposition of the materials. All study materials returned to the Sponsor or destroyed will first be copied onto electronic media and the electronic copy will be retained in the BioReliance archives for a minimum of 10 years.

Raw data, the protocol and reports generated at facilities other than BioReliance will be archived per the contractual arrangements between that facility and the Sponsor.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE/TESTING GUIDELINES

This protocol has been written to comply with the testing guidelines:

OECD Guideline 474 (Genetic Toxicology: Mammalian Erythrocytes Micronucleus Test) and Guideline 475 (Mammalian Bone Marrow Chromosome Aberration Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998.
European Commission Directive 2000/32/EC of May 19, 2000, Annex 4C-B12. Mutagenicity – *In Vivo* Mammalian Erythrocyte Micronucleus Test. No. L 136.

U.S. Environmental Protection Agency (EPA), Health Effects test Guidelines OPPTS 870.5395, Mammalian Erythrocyte Micronucleus Test (August 5, 1998) and OPPTS 870.5385, Mammalian Bone Marrow Chromosome Aberration Test (August 5, 1998.

Portion of this study performed at BioReliance will be conducted in compliance with the provisions of the US FDA GLP Regulations 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 160 and 40 CFR 792, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice, as applicable to the product being tested.

An in-process phase, the raw data, and report(s) will be inspected per the Standard Operating Procedures (SOPs) of BioReliance by the Quality Assurance Unit of BioReliance for compliance with GLPs, the SOPs of BioReliance and the study protocol. At least one, study-specific, in-process inspection will be performed for this study. A signed QA statement will be included in the final report. This statement will list the

study-specific phases inspected, the dates of each inspection, and the dates the results of each inspection were reported to the Study Director and the Study Director's management. In addition, a signed GLP compliance statement will be included in the final report. This statement will cite the GLP guideline(s) with which the study is compliant and any exceptions to this compliance, if applicable, including the omission of characterization or stability analyses of the test substance or its mixtures.

Raw data, the protocol and reports generated at facilities other than BioReliance will be QA audited per the contractual arrangements between that facility and the Sponsor.

Alterations of this protocol may be made as the study progresses. All protocol modifications and rationale for the change(s) will be documented, signed, dated and approved by the Study Director, BioReliance QA and the Sponsor. All protocol amendments will be delivered to the Sponsor and all Principal Investigators (if any) via mail, electronic file transfer or fax transmission, as well as internally at the Test Facility, on or as close as possible to the effective date of the amendment.

Deviations from the protocol (i.e., unplanned changes) will be documented in a deviation report or a note to file will be generated. A deviation report will be signed by the Study Director and BioReliance QA. All deviations will be identified in the study report.

13.0 REFERENCES

Heddle, J.A. 1973. A rapid in vivo test for chromosomal damage. Mutation Res. 18:187-190.

Heddle, J.A., M. Hite, B. Kirkhart, K. Mavournin, J.T. MacGregor, G.W. Newell, and M. Salamone. 1983. The induction of micronuclei as a measure of genotoxicity. A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutation Res. 123:61-118.

Hayashi, M., R.R. Tice, J.T. Macgregor, D. Anderson, D.H. Blakey, M. Dirsch-Volders, F.G. Oleson Jr., F. Pacchierotti, F. Romagna, H. Shimada, S. Sutou and B. Vannier. 1994. In vivo rodent erythrocyte micronucleus assay. Mutation Res. 312: 293-304.

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. S2A document recommended for adoption at step 4 of the ICH process on July 19, 1995. Federal Register 61:18198-18202, April 24, 1996.

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals. S2B document recommended for adoption at

step 4 of the ICH process on July 16, 1997. Federal Register 62:16026-16030, November 21, 1997.

Kastenbaum, M.A., and Bowman, K.O. 1970. Tables for determining the statistical significance of mutation frequencies. Mutation Res. 9:527-549.

Mavournin, K.H., D.H. Blakey, M.C. Cimino, M.F. Salamone and J.A. Heddle. 1990. The *in vivo* micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutation Res. 239:29-80.

OECD Guideline for the Testing of Chemicals, OECD Guideline 474 (Genetic Toxicology: Mammalian Erythrocytes Micronucleus Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998.

OECD Guideline for the Testing of Chemicals, OECD Guideline 475 (Mammalian Bone Marrow Chromosome Aberration Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998.

14.0 APPROVAL

Maria Donner, Ph.D. Sponsor Representative	04-JUN-2007 Date
BioReliance Study Director	11 June 2007 Date
Valentine O. Wagner, III for SA BioReliance Study Management	11 Jun 2007 Date

9.3 Appendix III: Certificate of Analysis

E. I. du Pont de Nemours and Company
Wilmington. DE 19898
USA

CERTIFICATE OF ANALYSIS

This Certificate of Analysis fulfills the requirement for characterization of a test substance prior to a study subject to GLP regulations. It documents the identity and content of the test substance. This work was conducted under EPA Good Laboratory Practice Standards (40 CFR 792).

Haskell Code Number	H-28072
Common Name	HFPO Dimer Acid Ammonium Salt
Purity Percent	82.6%
Other Components	Water – 13.9% Ammonium (excess) – 3.5%
Date of Analysis	July 19, 2007
Recommended reanalysis interval	1 year
Instructions for storage	NRT&H
Reference	DuPont-23285
Analysis performed at	E. I. DuPont de Nemours and Company DuPont Haskell Laboratories Newark, Delaware USA

Peter A. Bloxham, Ph.D.
Analyst's Name

Analyst's signature

23-JULY-2007
Date

Revision #1
July 20, 2007